



ATTORNEY DOCKET NO. 014028.0290
SERIAL NO. 09/389,565

RECEIVED
APR 12 2001
TECH CENTER 1600/2900

IN THE SPECIFICATION

On page 1as amended by the Continuing Application Transmittal Form, please replace the first sentence stating priority with the following statement:

C'
--This application is a continuation of, and claims the benefit of, Serial No. 08/739,703, filed October 29, 1996, which status is abandoned, and which application is hereby incorporated herein by reference, and claims the benefit of priority of provisional application Serial No. 60/008,104, filed October 30, 1995.--

RECEIVED
APR 12 2001
TECH CENTER 1600/2900

On page 41-42, please replace the bridging paragraph with the following replacement paragraph:

C²
--The recombinant immunotoxin, sFv-DT390, was generated in two phases. First the coding sequences for the variable light (V_L) and variable heavy (V_H) chain regions of the UCHT1 antibody were amplified by a two step protocol of RT-PCR using primers based on the published sequence (13). The 5' V_L primer added a unique NcoI restriction enzyme site while the 3' V_H primer added a termination codon at the J to constant region junction and an EcoRI site. The V_L region was joined to the V_H region by single-stranded overlap extension and the two regions are separated by a (Gly₄Ser)₃ (SEQ ID NO:15) linker that should allow for proper folding of the individual variable domains to form a

C2
ent

function antibody binding site (14). Second, genomic DNA was isolated from a strain of *C. diphtheriae* producing the DT mutant CRM9 (C7[$\beta^{htox-201tox-9h'}$]) as described (15). This DNA was used for PCR. The 5' primer was specific for the toxin gene beginning at the signal sequence and added a unique NdeI restriction site. The 3' primer was specific for the DT sequence terminating at amino acid 390 and added an NcoI site in frame with the coding sequence. The PCR products were digested with the appropriate restriction enzymes and cloned into the *E. coli* expression plasmid pET-17b (Novagen, Inc., Madison, WI, USA) which had been linearized with NdeI and EcoRI. The resulting plasmid was used to transform *E. coli* BL21/DE3 cells. Cells were grown to an OD₅₉₀ of 0.5, induced with 0.5 M IPTG (Invitrogen, San Diego, CA, USA) and incubated for an additional 3 hours. The sFv-DT390 protein was isolated in the soluble fraction after cells were broken with a French Press and the lysate subjected to centrifugation at 35,000 X g.--

On page 49, replace the full paragraph with the following replacement paragraph:

C3

--Having observed that the epitope(s) recognized by the antibodies important for protection lay in the C-terminal 150 amino acids, a single-chain immunotoxin was generated with the first 390 amino acids (out of 535) of DT. Position 390 was chosen for 2 reasons: first, the 3 dimensional structure of DT suggested that this position was an external point on the molecule away from the

C3
CMT

enzymatic domain (18), and second, fusion toxins have been generated with longer DT subfragments with no reports of serum effects (19). The DNA encoding the first 390 amino acids of DT was ligated to DNA encoding the anti-CD3esFv (V_L linked to V_H using a $(Gly_4Ser)_3$ (SEQ ID NO:15) linker sequence). The predicted molecular weight for the fusion protein is 71,000 Daltons and has been confirmed by Western Blot analysis of both *in vitro* transcribed and translated protein as well as protein isolated from *E. coli* using goat anti-DT antibodies. The toxicity of sFv-DT390 protein, isolated from *E. coli* strain BL21/DE3, was compared to UCHT1-CRM9 in protein synthesis inhibition assays (Figure 3A). The IC_{50} (concentration required to inhibit protein synthesis to 50% of controls) of sFv-DT390 was 4.8×10^{-11} M compared to 2.9×10^{-12} M for UCHT1-CRM9, a 16-fold difference. To demonstrate the specificity of the sFv-DT390 construct, competition experiments were performed using increasing concentrations of UCHT1 antibody as competitor (Figure 3B). The results showed that approximately 1/8 antibody is needed to compete the sFv-DT390 toxicity to 50% as compared to UCHT1-CRM9. The antibody was capable of totally competing toxicity of both constructs thereby showing their specificity. The immunotoxins were then subjected to protein synthesis assays in the presence of increasing dilutions of serum (Table 5).--

On page 56, replace the full paragraph with the following replacement paragraph:

C4

--Primers used for the antibody engineering are listed in Table 6, and the primer sequences are based on published data [13]. The procedures of cloning scUCHT1 is schematically depicted in Fig. 4. mRNA isolated from UCHT1 hybridoma cells (provided by Dr. P. C. Beverley, Imperial Cancer Research Fund, London) was reverse transcribed into cDNA. The V_L and V_H regions of UCHT1 were amplified with polymerase chain reaction (PCR) from the cDNA using primer pairs P1, P2 and P3, P4 respectively. Primers P2 and P3 have a 25 bp complementary overlap and each encoded a part of a linker peptide (Gly₄Ser)₃. The single chain variable fragment (V_L -linker- V_H) was created by recombinant amplification of V_L and V_H using primers P1 and P4. A mouse kappa chain signal sequence was added at the V_L 5'-end by PCR, first with primers SP2 and P4, and then with primers SP1 and P4. The human IgM Fc region (CH₂ to CH₄) was amplified from the plasmid pBlue-huIgM (kindly provided by Dr. S. V. S. Kashmiri, National Cancer Institute, Bethesda). This gene fragment was about 1.8 kb. The V_L -linker- V_H -CH₂ region which is important for antigen recognition was confirmed by sequence analysis. Finally, the single chain variable fragment and the human IgM Fc region were cloned into plasmid pBK/CMV (Stratagene, La Jolla, CA, USA). Using the generated pBK/scUCHT1 plasmid as template, an *in vitro* transcription/translation assay yielded a product of 75 kDa, the expected size.--